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## Rapid Publication

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# Decreased Apoptosis in Proliferative and Postmitotic Regions of the Caspase 3-Deficient Embryonic Central Nervous System

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### ABSTRACT

*Caspase 3* (CPP32/Yama/apopain), a mammalian homolog of the *Caenorhabditis elegans* pro-cell death gene *ced-3*, is required for normal programmed cell death (PCD) in the nematode. Its prior deletion by homologous recombination in mice resulted in embryonic/early postnatal lethality associated with dramatic central nervous system (CNS) hyperplasia, yet a reported subtle decrease in cell death (Kuida et al. [1996] *Nature* 384:368–372). By comparison, the magnitude and distribution of dying cells identified using a DNA end-labeling technique, in situ end-labeling plus (ISEL+) (Blaschke et al. [1996] *Development* 122:1165–1174; Blaschke et al. [1998] *J. Comp. Neurol.* 396:39–50), supported an alternative explanation where the loss of caspase 3 function produces a more pervasive block in cell death, particularly among neuroblasts. To determine the relationship between loss of caspase 3 and dying cells identified by ISEL+, we analyzed caspase 3 +/+, +/-, and -/- embryos for normal caspase 3 expression and ISEL+ labeling. Both caspase 3 mRNA and active caspase 3 protein are present throughout the +/+ embryonic CNS, and both are absent from -/- embryonic cortices. Quantitation of dying cells identified by ISEL+ reveals a 30% reduction of labeled cells throughout the caspase 3 -/- embryonic cortices relative to +/+ littermates. Associated with this decrease is marked expansion of the total population of actively proliferating neuroblasts identified by 5-bromo-2'-deoxyuridine incorporation that nevertheless appears to maintain histological features of normal neurogenesis rather than dysregulated, neoplastic growth. These data indicate that caspase 3 deficiency results in a pervasive, albeit partial, decrease in embryonic neuroblast apoptosis that

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can account for the observed phenotypic hyperplasia in  $-/-$  embryos, and support the additional operation of caspase 3-independent PCD mechanisms during embryonic CNS development. *J. Comp. Neurol.* 423:1–12, 2000. © 2000 Wiley-Liss, Inc.

**Indexing terms:** brain development; ISEL+; mice; programmed cell death; ventricular zone

Programmed cell death (PCD) is a genetically regulated mechanism that plays a critical role during development and homeostasis of multicellular organisms (Kerr et al., 1972; Ellis et al., 1991). Apoptosis is the best known morphological type of PCD (Kerr et al., 1994) and is characterized by DNA fragmentation at internucleosomal sites (Wyllie et al., 1984). Programmed cell death has been studied for many years in relation to the development of the central nervous system (CNS). Most studies of PCD in neuronal development have focused on the period of target innervation, when differentiating neurons are sending out axons and making synaptic connections. This “target-dependent” PCD is thought to be involved in the size-matching of neuronal populations with their synaptic targets; it has been shown that neurons are overproduced during early development, and that appropriate neuronal numbers and connections are generated through selective PCD at a later time (Clarke, 1985; Oppenheim, 1985).

More recent studies, however, have shown that the period of target innervation is not the only period in development in which PCD is significant. Throughout the developing CNS, studies using a DNA end-labeling technique, in situ end-labeling plus (ISEL+), which identifies dying cells in situ, have shown that a large percentage of cells in proliferative regions also undergo PCD (Blaschke et al., 1996, 1998). These cells have not yet sent out axons to their synaptic targets, and therefore classical, target-dependent mechanisms cannot account for this death. Consistent with the operation of PCD during proliferative stages of CNS development, genetic deletion of key pro-cell death genes such as *caspases 3, 9*, and *Apaf-1*, has also revealed early defects in the CNS. Caspase 3/PPP32/yama/apopain (Kuida et al., 1996) was originally described as the protease responsible for poly (ADP-ribose) polymerase (PARP) cleavage during apoptosis in thymocytes (Nicholson et al., 1995). However, when the *caspase 3* gene was deleted in mice, there was no discernible phenotype in the immune system, whereas the CNS showed massive hyperplasia, with distortions in the cytoarchitecture visible even by embryonic day 12 (E12). The phenotype was attributed to decreased cell death (Kuida et al., 1996), although the reported mild and anatomically localized decrease was not consistent with the overall degree of pervasive hyperplasia observed, leaving open the possibility that increased cell number was due to oncogenic effects rather than decreased cell death. Adding to this uncertainty is the unknown location of *caspase 3* gene expression and active caspase 3 protein expression in the CNS, knowledge that could identify cells that would probably be affected by the null mutation. Here we report the normal mRNA and protein expression pattern of caspase 3 in  $+/+$  and mutant murine CNS during development and its relationship to cell proliferation and PCD.

## MATERIALS AND METHODS

### Animal and tissue collection

All animal protocols have been approved by the Animal Subjects Committee at the University of California, San Diego, and conform to National Institutes of Health guidelines and public law. Three pregnant caspase 3  $+/-$  heterozygote mice were received from the Section of Immunobiology, Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT. They were injected intraperitoneally with 20  $\mu$ l/g body weight of a 10 mM 5-bromo-2'-deoxyuridine (BrdU; Sigma, St. Louis, MO) solution in saline 1 hour before sacrifice. They were sacrificed in order to obtain three litters at age E12 (litters 1–3), which were used for in situ hybridization analysis, and cell death and proliferation studies. We also expanded our own colony at the University of California, San Diego animal facility starting from one male and three female caspase 3  $+/-$  heterozygotes. One of these litters was analyzed at E12 by Western blot.

Genotyping was done by Southern blot (see below), using fragments of tails (at postnatal age) or the lower part of the body (at embryonic age) as the DNA source. The embryos were obtained at almost the expected Mendelian ratio  $+/+:+/-:-/-$  1:2:1 (4:11:6 for the three original litters).

Balb/c mice (Simonsen, Gilroy, CA) were also used for analyzing caspase 3 expression in the brain during ontogenesis, and various organs in the adult, by using Northern and Western blot, and in situ hybridization studies. Timed-pregnant female mice were killed by cervical dislocation, and the embryos were removed at the appropriate age (E12–E18). For Northern and Western blot analysis, brains were removed from the embryo under a microscope. Postnatal mice at various ages (P0–P31) were sacrificed by decapitation, and their brains were quickly removed. Adult male mice were killed by cervical dislocation, and the brain and various organs were quickly removed.

### Southern blot analysis

Tails and lower extremities were collected from all embryos of the caspase 3 mutant litters obtained from heterozygote matings. Tissue was digested in 75 mM NaCl, 25 mM ethylenediaminetetraacetic acid (EDTA), 1% sodium dodecyl sulfate (SDS), 10 mM Tris-HCl, pH 8.0, and 0.4 mg/ml proteinase K (added fresh) at 50°C overnight (o.n.). The DNA was recovered by phenol/chloroform extraction, and 20  $\mu$ g was digested o.n. using the *EcoRV* restriction enzyme, run on a 0.8% agarose gel, acid-nicked in 0.24 N HCl for 15 minutes, and then reequilibrated in 0.4 N NaOH for 30 minutes. The DNA was then transferred to nylon filters (Gene Screen Plus, DuPont/NEN, Boston, MA) in 0.4 N NaOH o.n. Blots were neutralized in 0.2 M Tris-HCl pH 7.5/2 $\times$  standard saline citrate (SSC; 1 $\times$  = 150 mM NaCl, 15 mM Na<sub>3</sub>citrate, pH 7.0) and crosslinked in a UV crosslinker (Gene Linker; Bio-Rad, Hercules, CA). Next, blots were prehybridized at 65°C in

hybridization buffer (5× SSC, 5× Denhardt's solution, 0.5% SDS, pH 7.3) and then probed o.n. in hybridization buffer containing the <sup>32</sup>P-labeled caspase 3 probes (1–5 × 10<sup>6</sup> cpm/ml hybridization buffer) and 100 μg/ml denatured sonicated salmon sperm DNA, at 65°C. The probes, containing the 831-bp open reading frame for caspase 3, were generated using random hexamer priming and [ $\alpha$ -<sup>32</sup>P]dCTP (DuPont/NEN). Blots were washed in 2× SSC/0.1% SDS two times for 15 minutes each, at room temperature (RT), 1× SSC/0.1% SDS for 15 minutes at RT, and 0.2× SSC/0.1% SDS for 15 minutes at 65°C and then exposed to Kodak XAR film (Kodak, Rochester, NY) at –80°C for 1 week with intensifying screens.

### Northern blot analysis

Total RNA from embryonic (E13–E18), postnatal (P7, P18, P31), or adult brains and adult spleen, thymus, and liver from Balb/c mice was extracted by following the standard protocol (Ausubel et al., 1994). Total RNA (20 μg) from each sample was electrophoresed through a 1% agarose-formaldehyde gel and transferred to a Hybond-N membrane (Amersham Pharmacia Biotech, Arlington Heights, IL). Blots were cross-linked by UV and prehybridized in hybridization buffer containing 25% v/v formamide, 0.5 M Na<sub>2</sub>HPO<sub>4</sub> pH 7.0, 1% bovine serum albumin (BSA), 1 mM EDTA, 5% SDS, at 55°C for at least 1 hour. <sup>32</sup>P-labeled caspase 3 probes, containing the entire open reading frame, were generated as described above. Blots were hybridized o.n. at 55°C in hybridization buffer containing probe at 5 × 10<sup>6</sup> cpm/ml and 100 μg/ml denatured sonicated salmon sperm DNA. Blots were washed in 2× SSC/0.1% SDS two times for 15 minutes each at RT, 1× SSC/0.1% SDS for 15 minutes at RT, 0.5× SSC/0.1% SDS for 15 minutes at RT, and 0.2× SSC/0.1% SDS for 15 minutes at 65°C. Blots were then exposed to Kodak XAR film at –80°C for 1–2 weeks with intensifying screens.

### In situ hybridization

Balb/c whole embryos, postnatal and adult mice (whole head or brain), and E12 caspase 3 mutant embryos were embedded in Tissue-Tek OCT (Optimal Cutting Temperature; Miles, Elkhart, IN) and quickly frozen on powdered dry ice. Sagittal sections were cut at 20 μm on a cryostat (Frigocut 2800E; Jung, Nussloch, Germany) and collected onto electrostatically charged slides (Superfrost Plus; Fisher, Pittsburgh, PA). Sections were fixed as described previously (Blaschke et al., 1996; Chun and Blaschke, 1997). Briefly, sections were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; 1× = 137 mM NaCl, 1.5 mM KCl, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, 2.7 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4), extracted with 0.6% w/v Triton X-100 in 2× standard sodium phosphate-EDTA (SSPE; 2× = 300 mM NaCl, 20 mM NaH<sub>2</sub>PO<sub>4</sub>, 25 mM EDTA, pH 7.4), acetylated, dehydrated through graded ethanols, and stored at –80°C until used.

Digoxigenin-labeled caspase 3 sense and antisense riboprobes were transcribed from the plasmid containing the full open reading frame of the *caspase 3* gene using standard protocols (Genius system; Boehringer Mannheim, Indianapolis, IN). In situ hybridization was performed as described previously (Weiner and Chun, 1997). Briefly, sections were hybridized o.n. at 65°C with sense or antisense riboprobe at a concentration of 0.25 ng/μl in in situ hybridization buffer, containing 50% v/v formamide, 2× SSPE, 10 mM dithiothreitol (DTT), 2 mg/ml yeast tRNA,

0.5 mg/ml polyadenylic acid, 2 mg/ml BSA, 0.5 mg/ml denatured salmon sperm DNA. The sections were then washed in 2× SSPE/0.6% w/v Triton X-100 two times for 45 minutes each and 1× high-stringency buffer (2 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1 mM EDTA, 1 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.0) at 65°C, three times for 30 minutes each. Sections were then rinsed in 1× Tris-buffered saline (TBS; 10× = 0.5 M Trizma base, 1.5 M NaCl, pH 7.5), blocked for 1 hour in 1% blocking reagent (Boehringer Mannheim) in 0.3% w/v Triton X-100, 1× TBS, and incubated o.n. in a 1:5,000 dilution of alkaline phosphatase-conjugated antidigoxigenin Fab fragments (Boehringer Mannheim) in the blocking reagent solution. Alkaline phosphatase activity was detected by incubation in substrate solution (100 mM Trizma base, 100 mM NaCl, 50 mM MgCl<sub>2</sub>, pH 9.5) containing 450 μg/ml 4-nitroblue tetrazolium chloride (NBT; Boehringer Mannheim) and 17.5 μg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Boehringer Mannheim). Sections were rinsed in Milli-Q H<sub>2</sub>O, counterstained with 4,6-diamino-2-phenylindole (DAPI; Sigma), mounted in Crystal Mount (Biomedica, Foster City, CA), and examined using a Zeiss microscope (Thornwood, NY).

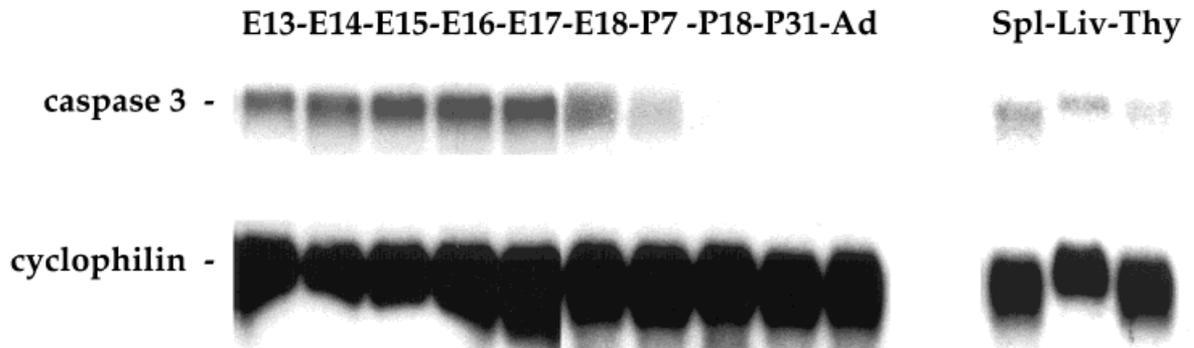
### Caspase 3 antisera/antibodies

Caspase 3 is generated as an inactive proenzyme that is activated by cleavage into p10 and p20 peptides (Salvesan and Dixit, 1997). In this study, we used two rabbit polyclonal antisera, CSP3 and CM1, which were obtained from IDUN Pharmaceuticals (La Jolla, CA), and one commercial mouse monoclonal antibody, cat. C76920, obtained from Transduction Laboratories (Lexington, KY). The CSP3 was obtained by immunization with a recombinant, processed caspase 3 and recognizes both the inactive p32 zymogen and the active p20 peptide (Srinivasan et al., 1998). The CM1 was obtained by immunization with a 13-amino acid peptide (CRGTELDCGIETD) corresponding to the C-terminus of the large subunit of the human and the mouse caspase 3 and recognizes only the active p20 peptide (Armstrong et al., 1997; Srinivasan et al., 1998). The commercial antibody recognizes both the mouse and the rat p32 zymogen.

### Western blot analyses

Proteins were extracted from several tissues: brains from embryonic, postnatal, or male adult Balb/c mice, various organs from adult male Balb/c mice, and brains from a litter of caspase 3 mutant E12 embryos. Proteins were obtained by homogenization of tissues in homogenization buffer, containing 20 mM Tris-HCl, pH 7.6, 250 mM NaCl, 3 mM EDTA, 3 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), and protease inhibitors (500 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 150 nM aprotinin, 1 μM E-64, 1 μM leupeptin; Calbiochem, San Diego, CA) in a dounce homogenizer (30 strokes) on ice. An equal volume of 2× lysis buffer (20 mM Tris-HCl, pH 7.6, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 40 mM β-glycerophosphate, 1% w/v NP-40) containing protease inhibitors was added to each sample. Samples were left on ice for about 1 hour and centrifuged at 14,000 rpm for 12 minutes at 4°C. The supernatant was recovered, and protein concentration was determined by using the Bradford method (Bio-Rad). Samples were stored at –80°C until used. Protein samples (20 μg) in Laemmli sample buffer (50% v/v glycerol, 0.5 M DTT, 10% SDS, 0.4 M Tris-HCl, pH 6.8) were boiled for 10

A



B

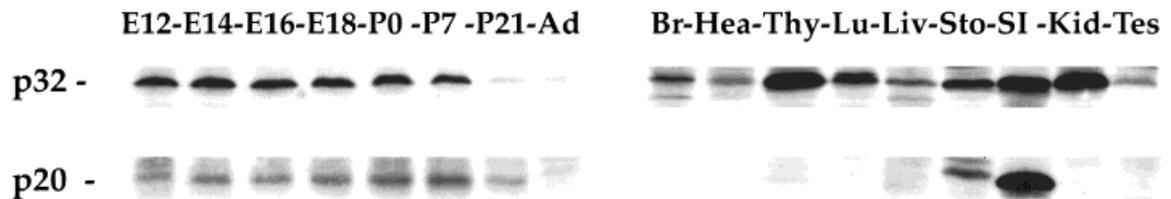


Fig. 1. Caspase 3 mRNA and protein are highly expressed in the embryonic mouse CNS and in some organs in adult mice. **A:** Northern blot analysis of total RNA from embryonic and postnatal Balb/c mouse brains, as well as some adult mouse organs. The full-length caspase 3 cDNA was used as a probe. The 2.6-kb caspase 3 mRNA was highly expressed throughout the embryonic period in the brain. Expression decreased significantly in the first postnatal week and was undetectable by adulthood. Caspase 3 mRNA was more highly expressed in the embryonic brain than in organs of the adult immune system, for example, the thymus, the organ from which caspase 3 was originally cloned. Blots were probed for the ubiquitously expressed cyclophilin gene as a loading control. **B:** Western blot analysis of protein (20  $\mu$ g, measured with the Bradford method) extracted from brains of Balb/c mice at several ages and from various organs from adult male mice.

The filters were probed with CSP3, a rabbit polyclonal antiserum that recognizes the unprocessed p32 zymogen form of caspase 3 (upper blots) and CM1, a rabbit polyclonal antiserum that recognizes the processed large p20 subunit of caspase 3 (lower blots). Expression in the CNS increased at later embryonic ages. The active zymogen was still very abundant at P7, whereas the unprocessed zymogen was decreasing. At later postnatal ages, both unprocessed and processed caspase 3 levels were very low. In the adult, several organs expressed high levels of the unprocessed zymogen, but only the small intestine, and to a lesser degree the stomach, showed high levels of the active protein. Ad, adult; Br, brain; E, embryonic day; Hea, heart; Kid, kidney; Liv, liver; Lu, lung; P, postnatal day; SI, small intestine; Spl, spleen; Sto, stomach; Tes, testis; Thy, thymus.

minutes and run on 15% SDS-polyacrylamide gel electrophoresis (PAGE) mini-gels, blotted onto PVDF membranes (Immobilon-P; Millipore, Bedford, MA), and blocked in 5% nonfat dry milk in PBS-T (1 $\times$  PBS, 0.1% w/v Tween-20) o.n. Blots were incubated with the caspase antiserum/antibody (CSP3 at 60 ng/ml, CM1 at 10 ng/ml, and C76920 at 25 ng/ml) in PBS-T for 2 hours, followed by three PBS-T washes and incubation with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse secondary antibody (Vector, Burlingame, CA) at 1:20,000 in PBS-T for 1 hour. After three more PBS-T washes, detection was achieved by using the ECL+Plus system (Amersham Pharmacia Biotech). After labeling with the caspase 3 antibodies, the blots from the caspase 3 mutant litter were stripped in a solution containing 100 mM  $\beta$ -mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8, 2% SDS at

65°C for 1 hour. They were washed in PBS-T and then reprobed with a rabbit polyclonal antibody against chicken synapsin I (1:10,000 for 2 hours) following the same protocol as above, as a loading and transfer control.

#### Detection of programmed cell death—ISEL+

The ISEL+ was performed on E12 embryos from three caspase 3 mutant litters, following the protocol described previously (Blaschke et al., 1996; Chun and Blaschke, 1997). Sagittal sections next in series to those used for in situ hybridization studies were cut at 10  $\mu$ m on the cryostat and collected on electrostatically charged slides. Sections were fixed as described above for in situ hybridization and incubated in labeling mix containing 100 mM potassium cacodylate, 2 mM CoCl<sub>2</sub>, 0.2 mM DTT, 0.5  $\mu$ M digoxigenin-11-2'-deoxyuridine-5'-

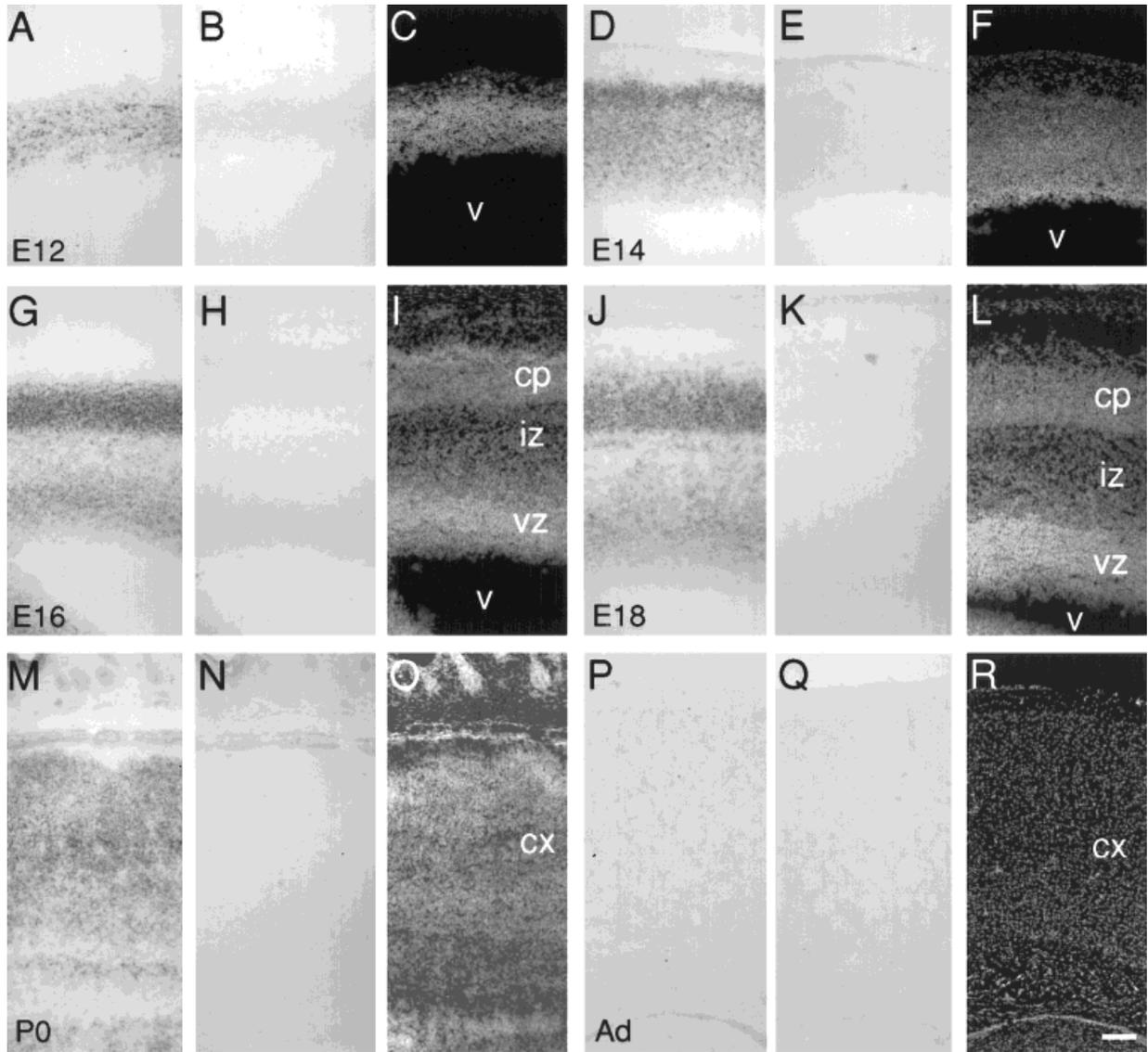


Fig. 2. Caspase 3 mRNA distribution in the embryonic CNS. In situ hybridization was performed on cryosections from the embryonic cerebral cortex obtained from animals at the following ages: E12 (A-C), E14 (D-F), E16 (G-I), E18 (J-L), P0 (M-O) and adult (P-R). Digoxigenin-labeled riboprobes transcribed in the antisense orientation from linearized plasmids containing the full-length caspase 3 cDNA were used as probes (A,D,G,J,M,P). Digoxigenin-labeled sense riboprobes were used as controls on serial tissue sections (B,E,H,K,N,Q). Counterstaining with the fluorescent nuclear dye 4,6-

diamino-2-phenylindole (DAPI) is shown for the sense hybridization slides (C,F,I,L,O,R). Caspase 3 hybridization signal at E12 was present throughout the cerebral wall. At E14, E16, and E18, caspase 3 expression was increased in the cortical plate but was still present throughout the cerebral wall. No hybridization signal was detected for the sense probe at any age. Ad, adult; cp, cortical plate; cx, cerebral cortex; E, embryonic day; iz, intermediate zone; P0, postnatal day 0; v, lateral ventricle; vz, ventricular zone. Scale bar in R = 100  $\mu$ m in A-O; 200  $\mu$ m in P-R.

monophosphate (dig-dUTP; Boehringer Mannheim), and 150 U/ml terminal deoxynucleotidyl transferase (TdT; Boehringer Mannheim) for 1 hour at 37°C. Endogenous phosphatases were inactivated by a 2-hour incubation in 2 $\times$  SSPE at 65°C. Incorporated digoxigenin was detected as described above for in situ hybridization, except that a 1:500 dilution of the alkaline phosphatase-conjugated antidigoxigenin Fab fragments (Boehringer Mannheim) was used. Slides were counterstained with DAPI and mounted in Crystal Mount.

### Detection of cell proliferation

Proliferating cells were detected by the incorporation of BrdU injected 1 hour before sacrifice in the mothers of the three litters examined (see above). Actively proliferating cells in S-phase will incorporate BrdU in their DNA, and the BrdU will be detectable by immunocytochemistry. The S-phase zone is located in the upper part of the ventricular zone (vz). Sections (10  $\mu$ m thick) next in series to those used for ISEL+ labeling were incubated o.n. at 65°C in 2 $\times$

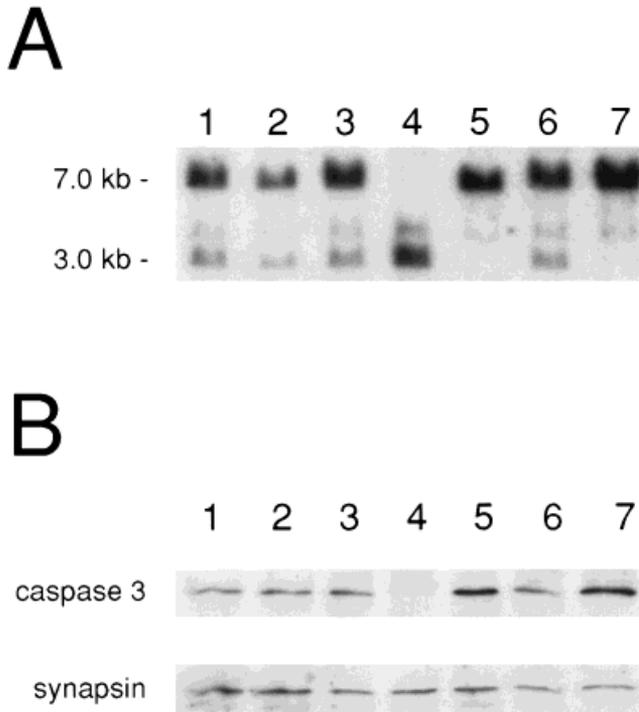


Fig. 3. Southern and Western blot analysis of a caspase 3 mutant litter. **A:** Southern blot analysis of tail DNA from embryos of litter 9. A full-length caspase 3 cDNA was used as a probe. This probe hybridized to a 7.0-kb band in the wild-type genome and to a 3.0-kb band in the mutant genome. Embryos #5 and 7 are homozygous wild-type, embryos #1–3 and 6 are heterozygotes, and embryo #4 is mutant. **B:** Western blot analysis of protein extracted from the brains of the embryos of litter 9, the same as above. The filter was probed with the CSP3 antiserum that recognizes the unprocessed p32 zymogen form of caspase 3 (upper blot). Caspase 3 was expressed at high levels in the homozygous wild-type embryos (#5 and 7) and at lower levels in the heterozygote embryos (#1–3 and 6). No caspase 3 was detectable in the mutant embryo (#4). The filter was reprobed with an antibody against synapsin I, as loading and transfer control (lower blot).

SSC/50% v/v formamide, washed in  $2\times$  SSC, incubated in 2 N HCl at  $37^{\circ}\text{C}$  for 30 minutes, neutralized in 0.1 M boric acid, pH 8.5, for 10 minutes, and rinsed in  $1\times$  PBS. Sections were then blocked for 1 hour in 2.5% BSA/0.3% w/v Triton X-100/PBS blocking buffer, pH 6.7, and incubated o.n. with anti-BrdU antibody (Boehringer Mannheim) at 6  $\mu\text{g}/\text{ml}$  in blocking buffer. Next, the sections were washed in PBS and incubated for 1 hour in a 1:200 dilution of biotinylated anti-mouse antibody (Vector) in PBS. Biotinylated antibody was detected by using an ABC peroxidase kit (Vector), with 3,3'-diaminobenzidine tetrahydrochloride (Sigma) as the chromogen. Slides were lightly counterstained with Cresyl Violet, dehydrated through graded ethanols and xylene, and mounted in Cytoseal (Stephens Scientific, Riverdale, NJ).

#### Double labeling with BrdU and ISEL+

Double labeling was performed by combining the ISEL+ and BrdU detection protocols as previously described (Blaschke et al., 1998; Pompeiano et al., 1998). Briefly, 10- $\mu\text{m}$  sections third in series to those used for ISEL+ and BrdU single labeling were end-labeled using TdT and dig-dUTP and incubated o.n. in  $2\times$  SSC/50% v/v formamide; then the ISEL+ protocol was completed. Following the ISEL+ color reaction, the BrdU detection protocol was completed, starting with the incubation in 2 N HCl at  $37^{\circ}\text{C}$  (see above).

#### Quantitation of dying and proliferating cells

Both dying and proliferating cells were counted in fields taken from the anteromedial part of the developing cerebral cortex. At this level, no abnormal folding of the developing cortex was observed in the caspase 3  $-/-$  embryos. Cell counts were performed on photographs taken at  $1,000\times$  with a Zeiss Planapo lens of both single- and double-labeled sections from all 21 embryos from the three caspase 3 mutant litters. Total dying cells were counted on at least two fields extending through the whole thickness of the cerebral cortex from each of the two ISEL+ single-labeled sections for each embryo. The same fields were

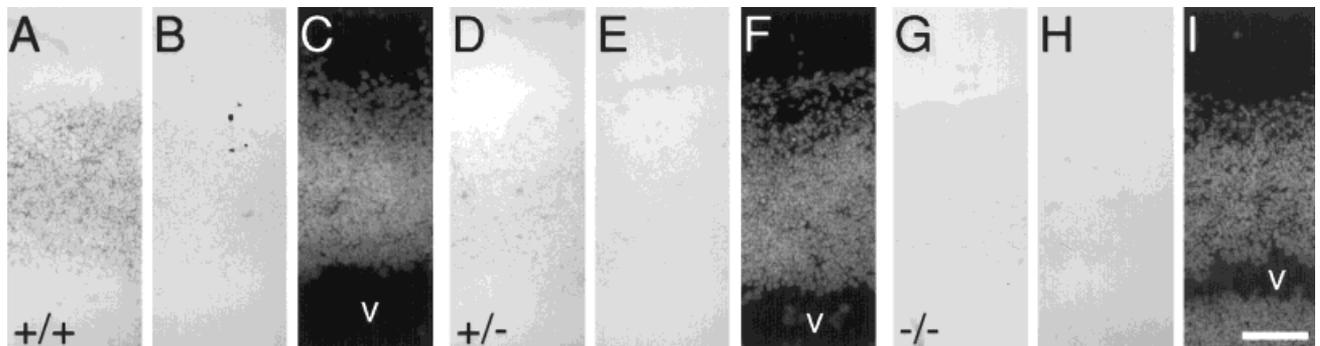


Fig. 4. Caspase 3 mRNA is absent in  $-/-$  mutant brains. In situ hybridization studies were performed on embryos of different genotypes from the three original litters. Caspase 3 expression is shown for three animals from litter 3: #4 (+/+, **A–C**), #2 (+/–, **D–F**), and #5 (–/–, **G–I**). Digoxigenin-labeled antisense riboprobes showed that caspase 3 mRNA was expressed in +/+ embryos (**A**). The hybridization signal was still present but at lower levels in +/- embryos (**D**). No

caspase 3 mRNA was detectable in the  $-/-$  embryos (**G**). Controls using digoxigenin-labeled sense riboprobes on the next sections in series to those used for the antisense probe showed no hybridization signal (**B,E,H**). Counterstaining with the fluorescent nuclear dye 4,6-diamino-2-phenylindole (DAPI) is shown for the antisense hybridization slides (**C,F,I**). v, lateral ventricle. Scale bar = 100  $\mu\text{m}$ .

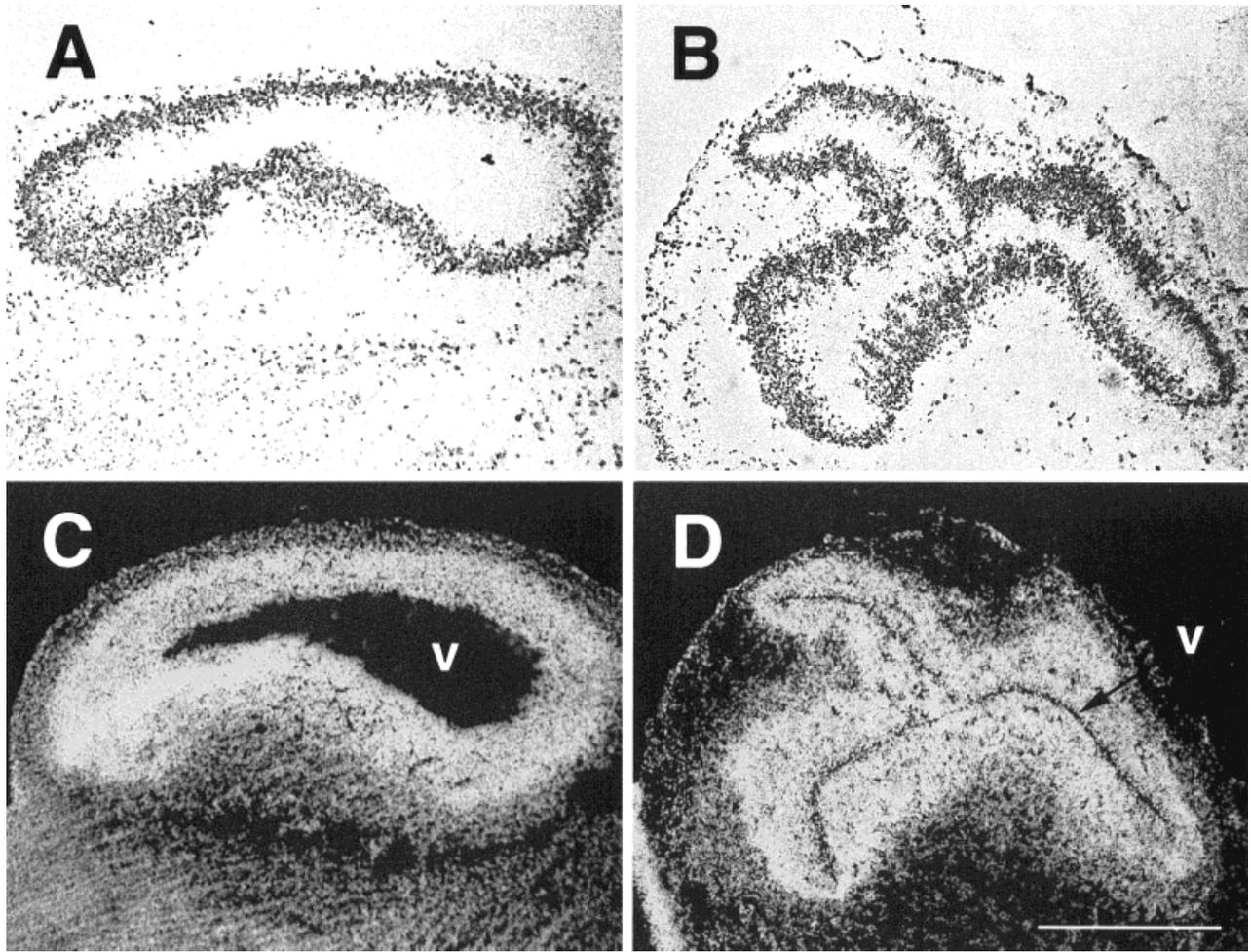


Fig. 5. Caspase 3  $-/-$  mutants have an expanded proliferative zone. **A,B:** 5-Bromo-2'-deoxyuridine (BrdU) immunolabeling in a section through the developing cerebral cortex of a heterozygote and mutant embryo, respectively. **C,D:** 4,6-Diamino-2-phenylindole (DAPI) staining of the next section in series from A and B, respectively. In the  $-/-$  mutant embryo, the cortex is distorted into folds

and the lateral ventricle is identifiable only as a thin slit in the center of the cortical mass. The thickness of the proliferative layer relative to the cortex looks very much alike in the two embryos. Statistics showed that the percentage of BrdU labeled cells is only slightly increased in the  $-/-$  with respect to the  $+/-$  embryos. v, lateral ventricle. Scale bar = 500  $\mu$ m.

photographed for ISEL+ labeling (bright field) and for DAPI counterstaining (fluorescent). Data are expressed as a percentage of ISEL+-positive cells over the total (DAPI stained). Proliferating cells were counted from two fields per embryo extending through the whole thickness of the cerebral cortex from the BrdU single-labeled sections. Data are expressed as a percentage of BrdU positive cells over the total (Cresyl Violet stained). Dying cells in the proliferative region were counted from one field for each embryo taken from the double-labeled sections. Data are expressed as a percentage of ISEL+/BrdU double-labeled cells over the total BrdU-labeled cells. Dying cells were also counted in postmitotic regions of the anterior cortex from ISEL+/BrdU double-labeled sections where BrdU labeling is used to define the upper limit of the proliferating vz. These counts were made from one to two fields for each embryo. Data are expressed as a percentage of ISEL+-positive cells over the total.

Data were combined for all animals within each genotype group. The differences in the proportions of labeled

and unlabeled cells were compared among the three genotypes using the G-test with Williams continuity correction (Sokal and Rohlf, 1995). When significant differences were detected, Gabriel's Simultaneous Test Procedure (Sokal and Rohlf, 1995) was used to determine which comparisons among the three genotypes were statistically significant.

### Figure production

All figures were generated by using Adobe Photoshop 5.5 and Adobe Illustrator 8.0 (Adobe Systems, Mountain View, CA) on an Apple Macintosh computer (Apple Computers, Cupertino, CA). For Figures 1 and 3, autoradiograms were scanned by using a UMAX scanner (UMAX Technologies, Fremont, CA). All other figures were obtained by scanning color negatives with a Nikon 35-mm film scanner (Nikon Corporation, Tokyo, Japan). Only minor corrections were made in the brightness and contrast of the images.

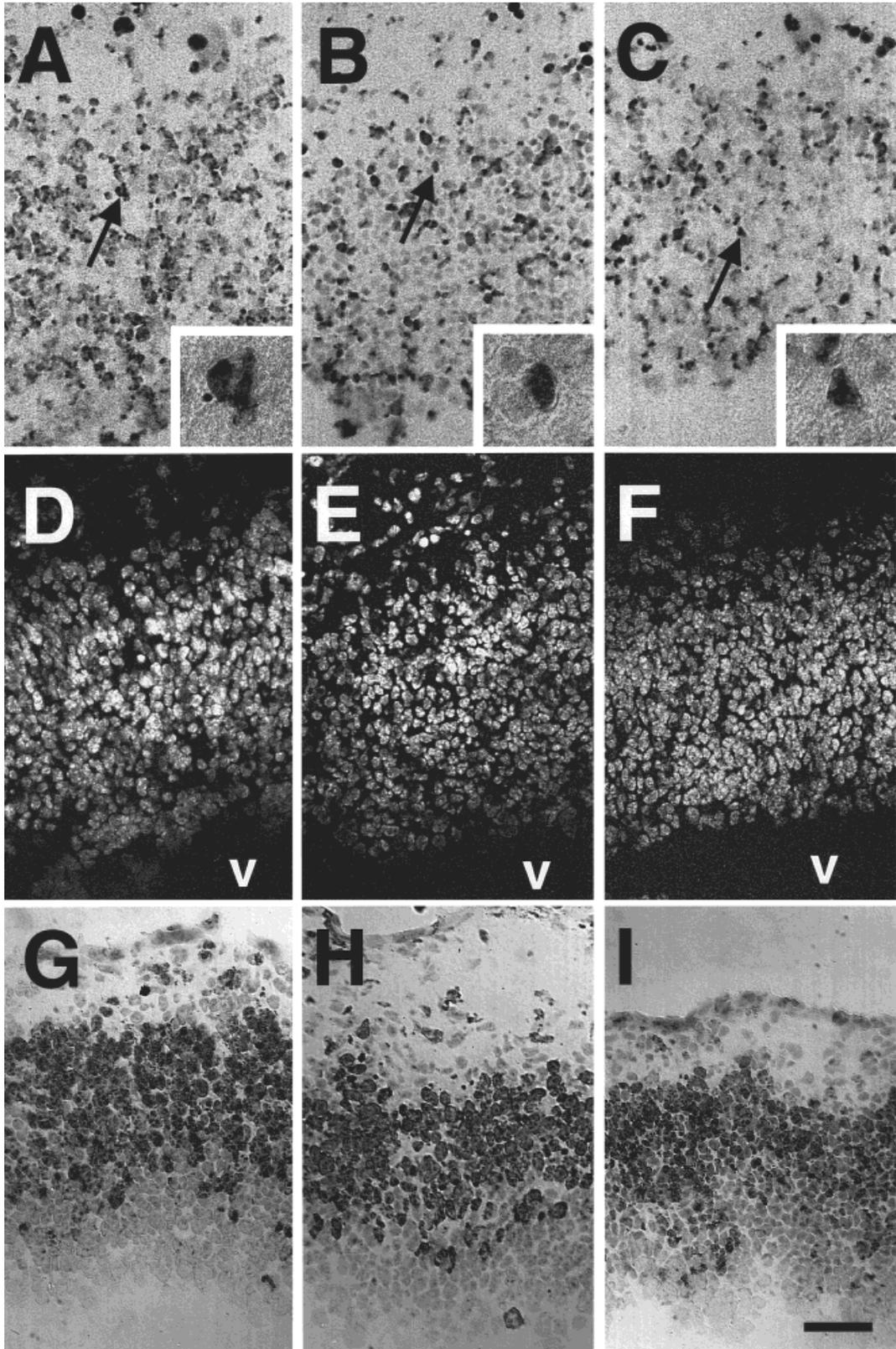


Figure 6

## RESULTS

### Caspase 3 expression in embryonic brain

The presence of caspase 3 mRNA and protein was examined in the murine CNS. Northern blot analysis of total RNA from embryonic and postnatal brains demonstrated that caspase 3 was highly expressed in the developing CNS (Fig. 1A). Caspase 3 mRNA levels were highest in the brain during embryonic life. There was, however, a slight increase from E13–E17, and then a decrease at E18. Caspase 3 mRNA decreased after E18, most significantly in the postnatal brains: it was still detectable at P7 but virtually undetectable in this assay at later ages through adulthood. In the organs examined from adult mice, caspase 3 mRNA was comparatively lower in the spleen and the liver and barely detectable in the thymus, the organ from which caspase 3 was originally cloned.

In situ hybridization studies provided spatial information about caspase 3 expression patterns within the developing cerebral cortex (Fig. 2). At E12, caspase 3 mRNA expression was evenly distributed throughout the cerebral wall (Fig. 2A). At E14, the signal increased in intensity, particularly in the superficial cerebral wall (Fig. 2D). At E16, caspase 3 expression in the postmitotic cortical plate was higher than in the proliferative vz (Fig. 2G). Some heavily labeled cells were also seen within the intermediate zone, and this pattern was also observed at E18 (Fig. 2J). At birth, caspase 3 in situ hybridization label was still pronounced throughout the cerebral wall (Fig. 2M), but decreased to low or undetectable levels in the adult cerebral cortex without preferential layer distribution (Fig. 2P). The active and inactive forms of caspase 3 were assessed by Western blot of embryonic, postnatal, and adult brain. The inactive zymogen (at about 32 kDa) detected by the CSP3 antiserum (Fig. 1B, upper blot), as well as the commercial antibody (not shown), was present in the CNS at E12 and was highest from E14 to P7. Caspase 3 precursor protein levels were strongly reduced at P21, and they were very low but detectable in the adult brain. The active protein (at about 20 kDa), identified by the CM1 antiserum (Fig. 1B, lower blot), was present in the CNS at

E12 until E18 and then decreased to low, but still detectable levels, at P21 and in the adult.

Caspase 3 expression was also examined by Western blot in various organs from adult Balb/c mice (Fig. 1B). Very high levels of the inactive zymogen were observed in the thymus, the small intestine, and the kidney (Fig. 1B, upper blot). Lower levels were seen in the brain, the lung, and the stomach, and the lowest levels were detected in the heart, the liver, and the testis. The active protein was very abundant in the small intestine, less so in the stomach and very low, if detectable, in all other organs (Fig. 1B, lower blot).

### Analysis of caspase 3-deficient mice

**Caspase 3 expression.** All litters were genotyped by Southern blot, as shown in Figure 3A. Western blot confirmed that no caspase 3 protein was detectable in the mutant embryo brain (Fig. 3B, #4). Slightly lower levels of caspase 3 were observed in the heterozygote embryos (Fig. 3B, #1–3, 6) compared with the wild-type (Fig. 3B, #5 and 7). As expected, caspase 3 mRNA was detectable by in situ hybridization in the brain of caspase 3  $+/+$  embryos with a pattern and abundance similar to that observed in Balb/c E12 embryos (Fig. 4A). Lower levels of expression, with a similar distribution, were detectable in caspase 3  $+/-$  embryos (Fig. 4D), whereas no caspase 3 mRNA was detectable in  $-/-$  embryos (Fig. 4G).

**Cell death and cell proliferation.** All 21 embryos from litters 1–3 were used for cell death studies. By external examination, the head of the mutants was obviously bigger than that of the littermates, as previously described (Kuida et al., 1996). The body size appeared otherwise normal. Defective brain development was clearly visible in all caspase 3  $-/-$  mutant mice by DAPI or Cresyl Violet staining of sections (Fig. 5). Because of the increased cortical cell mass within the skulls of caspase 3  $-/-$  mutant mice, the cortex was distorted by several abnormal folds. The extent of the cerebral cortex appeared to be much greater in  $-/-$  mutant E12 embryos compared with littermates, as judged by simply looking at the expansion of the cerebral cortex in the parasagittal plane. The lateral ventricle, normally much larger than the cortex itself, as shown in heterozygotes (Fig. 5A,C), was visible as only a thin slit in caspase 3 mutant mice (Fig. 5B,D).

Based on the knowledge that *caspase 3* is a pro-cell death gene, the most likely explanation for the hyperplastic phenotype of caspase 3  $-/-$  mice was decreased PCD throughout the CNS. To test this hypothesis, ISEL+ was used to investigate cell death within the cerebral cortex of E12 embryos from 3 litters. ISEL+ identified a significant number of dying cells within  $-/-$  embryo cortices (Fig. 6C); however, a decrease in ISEL+-labeled cells was clearly visible in the cerebral cortex from  $-/-$  embryos compared with both  $+/+$  (Fig. 6A) and  $+/-$  littermates (Fig. 6B). Dying cells identified by ISEL+ were quantified in the whole thickness of the cerebral cortex from all embryos, and the values obtained for the percentage of dying cells over total cells in the examined fields are reported in Table 1. There was a significant difference in the labeled cell proportions among the three genotypes (corrected G value = 225.623,  $df = 2$ ,  $P < 0.001$ ). The percentage of labeled cells in  $+/+$ ,  $+/-$ , and  $-/-$  embryos were all significantly different from each other (Gabriel's Simultaneous Test Procedure,  $P < 0.05$ ). The percentage

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Fig. 6. Caspase 3  $-/-$  mutant embryos show decreased programmed cell death (PCD) in the cerebral cortex without dysregulated cell proliferation. Sections from wild-type, heterozygote or caspase 3  $-/-$  mutant embryos were processed with in situ end-labeling plus (ISEL+) to identify cells undergoing PCD (A,B,C, respectively) and counterstained with 4,6-diamino-2-phenylindole (DAPI) to show all cell nuclei (D,E,F, respectively). In A–C the arrows indicate an ISEL+-labeled cell that is enlarged in the inset. Sections from wild-type, heterozygote or caspase 3  $-/-$  mutant embryos were also processed with 5-bromo-2'-deoxyuridine (BrdU) immunohistochemistry to show proliferating cells (G,H,I, respectively). The ISEL+ labeling in wild-type embryos (A) showed approximately 50% of the cells undergoing PCD, a similar percentage to that seen in Balb/c embryos (Blaschke et al., 1996). In heterozygote embryos (B), 45% of cells were undergoing PCD (statistically significant compared with wild-type embryos). The percentage of dying cells was markedly lower (35% of total cells) in  $-/-$  mutant embryos (C). A 1-hour pulse of BrdU was administered before sacrifice and proliferating cells, which incorporated BrdU, were detected with BrdU immunohistochemistry. Tissue sections were counterstained with Cresyl Violet. BrdU labeling appeared very much alike in wild-type (G), heterozygote (H) and  $-/-$  mutant embryos (I). However, the proliferative zone in the mutants is expanded overall, in concert with the overall increase in the cortical surface. v, lateral ventricle. Scale bar = 50  $\mu$ m.

TABLE 1. Summary of the Results Obtained by Counting ISEL+, BrdU-, or ISEL+/BrdU Double-Labeled Cells in E12 Embryos from Three Mutant Litters<sup>1</sup>

	+/+ (n = 4)	+/- (n = 11)	-/- (n = 6)
ISEL+-labeled cells over total cells	49.6 (3,226)	45.2* (12,306)	36.5*** (8,414)
ISEL+-labeled cells over total cells (only postmitotic regions)	54.6 (767)	50.1 (2,463)	30.2*** (1,717)
BrdU-labeled cells over total cells	41.7 (3,895)	40.3 (10,969)	43.4** (7,153)
Double-labeled cells over BrdU-labeled cells	52.9 (1,120)	51.8 (6,133)	35.8*** (4,663)

<sup>1</sup> Note that only the actively proliferating cells in S-phase located in the upper part of the ventricular zone, not the entire ventricular zone, are labeled with a 1-hour BrdU pulse. Data are expressed as percentages, with total numbers of cells counted (labeled and unlabeled) in parentheses. Statistics were calculated using the numbers of labeled and unlabeled cells counted for each genotype.

\*Statistically significant difference with respect to the wild-type.

\*\*Statistically significant difference with respect to the heterozygote.

of dying cells was decreased by an average of 32% in  $-/-$  compared with  $+/+$  embryos. The decrease of dying cells in  $+/-$  with respect to  $+/+$  was about 10%.

To exclude the possibility that the decrease in the percentage of dying cells in  $-/-$  embryos could be due to an oncogenic, dysregulated increase in cell proliferation, we examined the population of proliferating cells as identified by BrdU incorporation. Pregnant  $+/-$  mice were injected with BrdU 1 hour before sacrifice. Using immunohistochemistry to detect BrdU-labeled cells, cell proliferation remained restricted to an S-phase zone in the upper part of the vz (Fig. 6G–I). No aberrant proliferation outside of the known proliferative regions was detected. We counted BrdU-labeled cells in the cerebral cortex of all 21 embryos from the three litters. A significant difference in the percentage of BrdU-labeled cells among the three genotypes was present (corrected G value = 16.813,  $df = 2$ ,  $P = 0.0002$ ). The percentage of labeled cells in  $-/-$  embryos was higher than in  $+/-$  embryos (Gabriel's Simultaneous Test Procedure,  $P < 0.05$ ), whereas all other comparisons were not statistically significant ( $P > 0.05$ , Table 1).

Because the hyperplastic phenotype of caspase 3  $-/-$  embryos was evident early in cortical development, when few neurons have become postmitotic, we previously hypothesized that caspase 3 might have increased importance for PCD among proliferating neuroblasts (Blaschke et al., 1996). To investigate this possibility, the percentage of ISEL+-labeled cells was counted separately in the proliferative compartment of the cerebral cortex as identified in double-labeled sections by combining ISEL+ with BrdU immunohistochemistry (Blaschke et al., 1998; Fig. 7). In these double-labeled sections, we also counted the percentage of ISEL+-labeled cells over the total in the forming postmitotic compartment.

The values obtained for the percentage of dying cells in the proliferative compartment, that is, the percentage of ISEL+/BrdU double-labeled cells over the total BrdU-labeled cells, are reported in Table 1. The values observed in wild-type embryos are consistent with previous observations (Blaschke et al., 1998). There were significant differences among labeled cell proportions for the three genotypes (corrected G value = 303.378,  $df = 2$ ,  $P < 0.001$ ). The percentage of double-labeled cells in  $-/-$  embryos was about 30% lower than in  $+/+$  and  $+/-$  embryos (Gabriel's Simultaneous Test Procedure,  $P < 0.05$ ), whereas the difference between  $+/+$  and  $+/-$  embryos was not statistically significant ( $P > 0.05$ ).

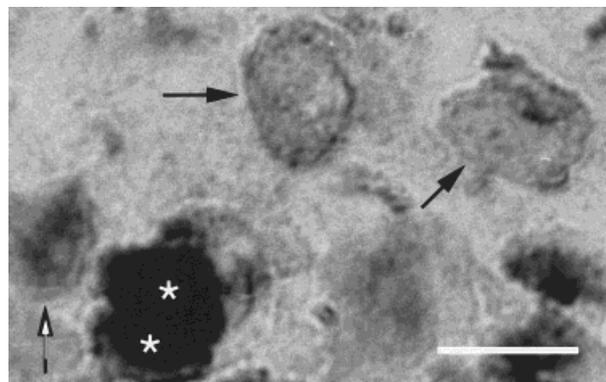


Fig. 7. Proliferating cells identified by 5-bromo-2'-deoxyuridine (BrdU) incorporation can be labeled by in situ end-labeling plus (ISEL+). Pregnant caspase 3  $+/-$  heterozygote mice carrying embryonic day 12 (E12) embryos were pulsed with BrdU 1 hour before being killed, and 10- $\mu$ m-thick sections of the E12 embryos were double-labeled with BrdU immunohistochemistry and ISEL+. A high magnification of the superficial part of the ventricular zone (S-phase zone) of a caspase 3  $+/-$  heterozygote embryo is shown. Black arrows point to proliferating cells labeled only with BrdU immunohistochemistry. White arrow with black outline points to a dying cell labeled only with ISEL+. Asterisks indicate cells that are double-labeled with both BrdU immunohistochemistry and ISEL+ (see also Blaschke et al., 1998). Scale bar = 10  $\mu$ m.

The percentages of dying cells in the postmitotic compartment are reported in Table 1. There were significant differences in the labeled cell proportions among the three genotypes (corrected G value = 209.475,  $df = 2$ ,  $P < 0.001$ ). The percentage of ISEL+-labeled cells in  $-/-$  embryos was significantly lower than in  $+/+$  and  $+/-$  embryos (Gabriel's Simultaneous Test Procedure,  $P < 0.05$ ), whereas the difference between  $+/+$  and  $+/-$  embryos was not statistically significant ( $P > 0.05$ ). The percentage of dying cells was decreased by about 40% in  $-/-$  with respect to both  $+/+$  and  $+/-$  embryos; however, the actual number of postmitotic neurons examined at this age is much smaller than that of the proliferating blasts.

## DISCUSSION

Programmed cell death is a genetically regulated mechanism for cellular suicide and plays a critical role in adult tissue homeostasis and during development. Much of our knowledge about the genes involved in regulating PCD comes from genetic studies in the nematode, *Caenorhabditis elegans* (Ellis et al., 1991). The mammalian homolog of CED-3, the product of the pro-apoptotic *ced-3* gene is represented by a highly conserved family of cysteine-containing, aspartate-specific proteases called caspases (Nicholson et al., 1995). The caspases are present in the cells as inactive proenzymes, and following a wide variety of apoptotic stimuli, they are activated by proteolytic cleavage into two subunits (Salvesan and Dixit, 1997). They are considered the key apoptotic executioners in mammals, and they mediate PCD by cleaving specific intracellular target proteins, including elements of the nucleus and the nuclear membrane, cytoskeleton, cytoplasm, and DNA, thus breaking down cell function and structure (Nicholson and Thornberry, 1997; Tan and Wang, 1998).

Our data showed that caspase 3 mRNA and protein are widely expressed and active in the developing CNS and are therefore likely to be important in the normal process of PCD on a global scale. The zymogen form of caspase 3 was found to be expressed at different levels in all the organs examined from adult mice. The active form was, however, detected only in the small intestine and at lower levels in the stomach. In the small intestine, caspase 3 was reported to be present in the cells lining the upper two-thirds of the villi (Krajewska et al., 1997). These findings are in excellent agreement with the high levels of apoptosis that we have observed in this tissue using ISEL+ and ligation-mediated PCR (Staley et al., 1997; Pompeiano et al., 1998).

Caspase 3 was expressed in the developing CNS at high levels early in cortical development and decreased significantly during perinatal life to low levels by adulthood. Caspase 3 expression was most apparent beginning around E12, when dying cells are prevalent in the cerebral cortex (Blaschke et al., 1996 and the present study). The hybridization signal was evenly distributed throughout the cerebral wall, consistent with the even distribution of ISEL+-labeled cells at this age (Blaschke et al., 1996 and the present study).

Litters of caspase 3 mutant mice were analyzed at E12 to avoid the cell death artifacts associated with brain compression that occurs in caspase 3  $-/-$  embryos at later ages. The number of dying cells as revealed using ISEL+ was reduced by about 30% in mutant with respect to the wild-type embryos and this reduction occurred throughout the cortex. The heterozygote embryos showed an intermediate phenotype, with a statistically significant reduction of cell death (about 10%). This result is in agreement with our *in situ* hybridization and Western blot results, showing that the expression levels of caspase 3 in  $+/-$  embryos was intermediate between those in  $+/+$  and  $-/-$  littermates. This decrease in PCD in  $+/-$  embryos was not accompanied by a detectable distortion of the cerebral cortex.

Clearly, a significant amount of PCD remains that must not be dependent on caspase 3 function. This is not unexpected in view of the multitude of other caspases along with the formal possibility of caspase-independent cell death. However, a decreased number of dying cells is seen in both postmitotic and proliferative compartments of caspase 3  $-/-$  embryos. The decreased number of dying cells in the proliferating regions probably accounts for the great increase in brain size observed even at E12, when most cells are still proliferating. As a result, for example, one "extra" proliferative cell present at E12 can become potentially scores of "extra" cells by the time of birth, whereas one "extra" postmitotic cell that does not undergo PCD when it should is simply one additional cell (Caviness et al., 1995; Takahashi et al., 1997).

We counted ISEL+ labeled cells in the whole thickness of the developing cerebral cortex and then separately in the postmitotic compartment (which at E12 is very small) and the proliferative compartment as identified by BrdU incorporation after a 1-hour BrdU pulse. (It is this S-phase zone that accounts for about 40% of the cells of the entire cerebral cortex; see Table 1.) The rest of the vz that extends substantially beneath the S-phase zone to the ventricle was not counted separately, because it probably contains nonproliferating cells in addition to proliferating cells (these latter cells would be labeled with BrdU using

a longer pulse or continuous BrdU administration). It is clear, however that a decrease of PCD is also found in this deeper region of the cortex that contributes to the overall values of ISEL+ labeling (Table 1) for the whole cortex compared with those within the S-phase zone alone. This effect was generally maintained (but with quantitative differences) in both the  $+/-$  and  $-/-$  genotypes. It is important to note that the decrease in ISEL+ labeling could formally be produced by an increase in the proliferative population of neuroblasts that was somehow independent of decreased PCD. Such a mechanism is inconsistent with what is known about caspase 3 function and is further inconsistent with the similar percentages of cells incorporating BrdU observed in all genotypes (Table 1). We conclude that the decrease in ISEL+ labeling reflects decreased PCD.

Our observations support the importance of caspase 3 in PCD throughout the developing brain. Mutation of caspase 3 primarily affects the CNS (Kuida et al., 1996); however, the caspase family is composed of at least 14 members (Cohen, 1997; Nuñez et al., 1998). It is thus probable that caspases other than caspase 3 may compensate for caspase 3 deletion, consistent with the decrease but not the disappearance of PCD in the developing cerebral cortex. Not mutually exclusive is the operation of caspase-independent death, as suggested by the continued loss of thymocytes in all examined caspase-null mutants (e.g., 3 and 9) (Kuida et al., 1996, 1998; Hakem et al., 1998).

What is the purpose of PCD in the embryonic cortex and nervous system? In the immune system, for example, thymic T-cells are selected—positively and negatively—for the expression of appropriate receptors (Shortman et al., 1990; Surh and Sprent, 1994) with inappropriate or dangerous T-cells undergoing PCD. Many cell death regulatory genes are used by both the immune and the nervous systems. Future work remains to be done to determine whether cell selection, in a manner analogous to that of the immune system, occurs in the nervous system, particularly in light of the recent data on neuronal DNA end-joining pathways used in immunological selection that affect neuronal PCD (reviewed in Chun and Schatz, 1999a,b).

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